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**Mutagenic Potential of Nitroguanidine  
in the Mouse Lymphoma Forward Mutation Assay**

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and  
Don W. Korte, Jr, PhD, MAJ MSC

Genetic and Cellular Toxicology Branch  
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Mutagenic Potential of Nitrosoguanidine in the Mouse Lymphoma Forward Mutation Assay--  
Harbell and Korte

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# ABSTRACT

The mutagenic potential of nitroguanidine (TP036) was assessed in the mouse lymphoma thymidine kinase forward mutation assay both with and without metabolic activation by rat liver S-9. In the initial range-finding assay, cells were exposed to test compound concentrations ranging from 4 mg/ml to 0.01 mg/ml. The confirmatory assay employed doses from 4 mg/ml to 1 mg/ml. Nitroguanidine did not induce a statistically significant increase in the mutant frequency in either of the two assays. These results indicate that nitroguanidine was not mutagenic under the conditions of this study.

Key Words: Mutagenicity, Genetic Toxicology, Mouse Lymphoma Assay, Nitroguanidine



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## PREFACE

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PROJECT/WORK UNIT/APC: #3E16270A835/180/TLB0

GLP STUDY NUMBER: 85035

STUDY DIRECTOR: MAJ Don W. Korte, Jr., PhD, MS

PRINCIPAL INVESTIGATOR: MAJ John W. Harbell, PhD, MS

REPORT AND DATA MANAGEMENT: A copy of the final report,  
retired SOPs, study protocol,  
retired stability and purity  
data on the test compound, and  
an aliquot of the test compound  
will be retained in the LAIR  
Archives.

TEST SUBSTANCE: Nitroguanidine CAS # 556-88-7

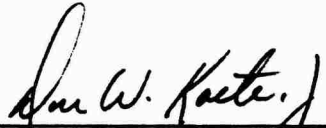
OBJECTIVE: The objective of this study was to determine the  
mutagenic potential of Nitroguanidine (TP036) by using the  
Mouse Lymphoma Forward Mutation Assay

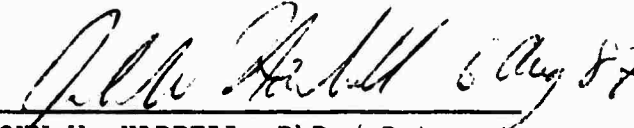
#### ACKNOWLEDGMENTS

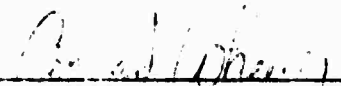
SGT Steven K. Sano and John Dacey provided research assistance during this study.

SIGNATURES OF PRINCIPAL SCIENTISTS AND MANAGERS INVOLVED IN  
THE STUDY

We, the undersigned, declare that GLP study number 85035  
was performed under our supervision, according to the  
procedures described herein, and that this report is an  
accurate record of the results obtained.

 2 DEC 87  
\_\_\_\_\_  
DON W. KORTE, JR, PhD / Date  
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 6 Aug 87  
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MEMORANDUM FOR RECORD

19 Nov 87

SUBJECT: Report of GLP Compliance for Study 85035

1. I hereby certify that the protocol for GLP Study 85035 was reviewed on 11 March 1985 and the study inspected 2 April 1985. The phase inspected was exposure of cells to compound.
2. The report entitled "Mutagenic Potential of Nitroguanidine (TPO36) in the Mouse Lymphoma Forward Mutation Assay," Toxicology Series 159, and the raw data were audited on 7 July 1987 and 5 November 1987.

*Carolyn M. Lewis*  
CAROLYN M. LEWIS  
C, Quality Assurance



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Mutagenic Potential of Nitroguanidine (TP036) in the Mouse  
Lymphoma Forward Mutation Assay -- Harbell and Korte

Nitroguanidine, a primary component of US Army triple-base propellants, is now produced in a Government-owned contractor-operated ammunition plant. The US Army Biomedical Research and Development Laboratory (USABRDL), as part of its mission to evaluate the environmental and health hazards of military-unique propellants generated by US Army munitions manufacturing facilities, conducted a review of the nitroguanidine database and identified significant gaps in the toxicity data (1). The Division of Toxicology, LAIR, was tasked by USABRDL to develop a genetic and mammalian toxicity profile for nitroguanidine, related intermediates/by-products of its manufacture, and its environmental degradation products.

Objective of the Study

The objective of this study was to determine the mutagenic potential of nitroguanidine (TP036) by using the Mouse Lymphoma Forward Mutation Assay.

MATERIALS AND METHODS

Nitroguanidine was evaluated for cytotoxicity and mutagenicity according to LAIR SOP, OP-STX-71 (2).

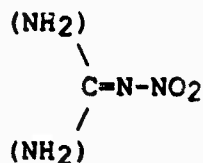
Test Compound

Chemical name: Nitroguanidine

LAIR Code name: TP036

Chemical Abstracts Service Registry No.: 556-88-7

Structural formula:



Empirical formula:  $\text{CH}_4\text{N}_4\text{O}_2$

Storage: Nitroguanidine was obtained from Hercules Aerospace Division, Sunflower Army Ammunition Plant, DeSoto, Kansas (lot no. SOW84k101 A001 for the initial and SOW83H001-004 for the confirmatory assay) and was assigned the LAIR

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Code number TP036. The test compound was stored at room temperature in a bunker on the Presidio of San Francisco. Small amounts of test compound were stored at room temperature in the laboratory just before use.

Chemical Properties/Analysis: Data characterizing the chemical composition and purity of the test material were prepared by the Division of Toxicology, LAIR, Presidio of San Francisco, CA (Appendix A).

#### Chemical Preparation

Nitroguanidine was dissolved directly in Fisher's Medium without serum with a final concentration of 4 mg/ml. This concentration is close to saturation for nitroguanidine in an aqueous medium (1).

#### Positive Controls

Ethyl methanesulfonate (EMS) (Sigma lot no. 83F-0279), added directly to the culture medium so as to provide a final concentration of 0.32 mg/ml, was used as the positive control for the assays conducted without metabolic activation. A stock solution of 2-acetamide fluorene (2AAF) (Sigma lot no. 113F-3679) in DMSO (Sigma lot no. 113F-0450) of 50 mg/ml (first assay) or 5 mg/ml (confirmatory assay) was prepared. One hundred microliters of this stock were used (0.5 or 0.05 mg/ml final concentration, respectively, in the two assays) as the positive control for assays conducted with metabolic activation. The final DMSO concentration of the 2AAF-treated cultures did not exceed 1%. Both positive controls were prepared fresh on the day of assay.

#### Cells

Mouse lymphoma cells L5178Y 3.7.2C TK<sup>+</sup>/ - were provided by Dr. Donald Clive, PhD, Burroughs Wellcome Co, Research Triangle Park, NC 27709. These cells were maintained in antibiotic-free Fisher's Medium for Leukemic Cells of Mice (Fisher's Medium) supplemented with 10% horse serum. Six days before each assay began, the cell population was cleared of spontaneous thymidine kinase negative mutants by methotrexate treatment (2) and screened for mycoplasma and other contaminants by using the 3T6 co-culture technique (3). No nonnuclear DNA was detected after four days of co-culture, and thus the cell line was presumed to be uncontaminated.

#### Medium

Powdered Fisher's Medium (basic) was purchased from Sigma Chemical Co (lot no. 113F-4710-1) and prepared in 10 mM HEPES buffered glass distilled water (pH 7.3). The medium

was immediately filter sterilized. The sterile medium was supplemented with l-glutamine (2 mM) and sodium pyruvate (1 mM). Sterile horse serum (lot no. 310437) was obtained from Sterile Systems Inc, Logan, Utah, and was heat inactivated (56°C for 30 minutes) before use. Fisher's Medium was supplemented with horse serum at 5%, 10%, or 20% (volume/volume) final concentration. These were designated F5p, F10p, and F20p, respectively, after the standard notation of Clive (4).

#### Metabolic Activation System

The metabolic activation system was composed of Aroclor-induced rat liver 9000 g supernatant fraction (S-9) and an NADPH-regenerating system provided by the cofactor mixture. Cofactor mixture, consisting of 2 mg/ml of NADP (Sigma lot no. 123F-7095 and 100F-7225) and 11.25 mg/ml of sodium isocitrate (Sigma lot no. 64F-3825), was prepared in Fisher's Medium without serum. In order to achieve the desired final nitroguanidine concentrations, cofactor mixture containing nitroguanidine was also prepared. This solution was prepared separately and mixed with normal cofactor mixture to obtain the desired concentration. These solutions were prepared immediately before use. When metabolic activation was used, 3 ml of cofactor solution were combined with 6 ml of cell suspension containing the treatment compound. Then 1 ml of S-9 was added to each group. Litton Aroclor-induced rat liver S-9 lot no. (RDK120) was used for each assay. Vials were thawed immediately before use.

#### Assay Format

**Dosing:** Stock cultures of L5178Y 3.7.2C cells were prepared for use by clearing spontaneous mutants and checking for contamination (see "Cells" above). Only cleared and noncontaminated cell populations were used for these assays. L5178Y cells were counted with a Coulter Counter model ZM (Coulter Electronic Inc, Hialeah, Florida) and resuspended in Fisher's Medium with 5% horse serum (F5p) at a concentration of  $10^6$  cells/ml. After one hour, 6 ml of the cell suspension were pipetted into each culture tube. The cells were then centrifuged down and the supernatant medium drawn off. The cells were then resuspended in F0p containing the desired concentration of nitroguanidine. For groups treated without metabolic activation, 9.7 ml were added, while those with metabolic activation received 5.7 ml. Three hundred microliters of horse serum were added to bring the serum concentration to 3%. Positive controls were added (see Tables 1 and 3 for concentrations). Negative controls were prepared for both the metabolic activation series and the nonactivation series. The groups of the metabolic activation series received 3 ml of cofactor mixture and 1 ml of freshly thawed S-9 suspension. The cofactor mixture also contained

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nitroguanidine in some cases to achieve the desired final compound concentration. The low serum (3%) concentration was intended to reduce the possible interaction (and inactivation) of test compounds with the serum proteins (4).

These cultures were maintained at 37°C on a roller drum for 4 hours, washed twice with Fisher's Medium containing 10% horse serum (F10p), resuspended in 20 ml of F10p, and returned to the roller drum. Ten percent serum in the medium provided for rapid growth in suspension culture.

Culturing: Approximately 24 hours after the cultures were first exposed, a sample of each culture was trypsin-treated for 10 minutes to produce a single cell suspension for counting. This suspension was then diluted to the appropriate concentration range and counted (average of three counts). The remaining cells from each culture were then diluted to  $3 \times 10^5$  cells/ml in 20 ml of F10p and returned to the roller drum. After approximately 48 hours, an aliquot from each culture was again counted. All cultures to be cloned at this point were diluted to  $3 \times 10^5$  cells/ml in Fisher's Medium with 20% horse serum (F20p). Twenty percent serum was used during cloning to enhance the absolute cloning efficiency.

#### Cloning

Nonselective: Soft agar cloning was used to determine the percentage of viable cells (viable count) and thymidine kinase negative mutants (mutant count) in each control and treated culture. To determine the percentage of viable cells, a portion of each freshly diluted culture ( $3 \times 10^5$  cells/ml) was further diluted to 600 cells/ml in F20p. One milliliter of this suspension was diluted in 105 ml of F20p containing 0.4% agar (Sigma lot no. 123F-0293) at 37°C. After vigorous mixing, this suspension of 5.7 cells/ml was dispensed into three 100 mm petri dishes (33 ml/dish). The extra 5 ml were provided to compensate for medium that foamed or adhered to the sides and thus could not readily be dispensed into the petri plates. The agar was allowed to harden at room temperature in the laminar flow hood (about 10 minutes).

Selective: To determine the percentage of thymidine kinase negative mutants, a similar but selective cloning procedure was performed. Ten milliliters of the  $3 \times 10^5$  cells/ml suspension were diluted with 95 ml of F20p with 0.4% agar (final concentration) which contained 1 µg/ml of trifluorothymidine (TFT) (Sigma lot no. 94F-0351). TFT was used to arrest the growth of all cells that contained thymidine kinase. After mixing, 33 ml of this  $2.86 \times 10^4$

cells/ml suspension were placed into each of three 100-mm petri dishes.

After hardening, both the mutant and viable count dishes were incubated for 11 days at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The number of colonies on each plate was then determined by using a Biotran II Automated Colony Counter (New Brunswick Scientific Co, Edison, New Jersey) with the size setting on zero.

#### Assay Acceptance Criteria

The following criteria are required, according to Brusick (5), for a valid assay.

Cloning Efficiency: The minimum negative control viable count cloning efficiency (mean count/mean number of cells plated) should be 70% or greater for the negative control cultures not treated with the activation mixture. A 100% cloning efficiency may be exceeded due to the delay between cell counts and dilutions during which the cells continue to divide. However, since the dilutions for the selective and nonselective cloning suspensions are made at the same time, the ratio between the two should not change even with the delay.

Cell Replication: The cells in the negative control cultures (without S-9 activation) should undergo at least a 15-fold increase in cell number over the two days of suspension culture. Negative control cultures treated with the metabolic activation mixture characteristically show slightly less growth and therefore may not undergo the 15-fold increase.

Positive Control Responses: A statistically significant mutagenic response (see below) must be induced by the positive controls. Failure to induce a mutagenic response by the positive activation control (2AAF) would invalidate only the activation series provided that the EMS (nonactivation control) induced an appropriate response.

Treatment Concentration: In the absence of strong mutagenic activity (e.g. possible nonmutagen), cells should be exposed to the test compound concentration to the limits of solubility (usually up to 5 mg/ml) or to the point where suspension growth is reduced by cytotoxicity to 10% of controls. In these assays, the solubility of nitroguanidine limited the upper dose concentration.

## DATA EVALUATION

Cell Replication and Survival

The combined activity of cell replication and survival for each control and treatment group is the product of suspension growth during the two days after exposure and the viable count cloning efficiency (2). Absolute suspension growth (ASG) is measured as a fold increase (usually 15- to 20-fold) in the control cultures over the 48-hour period. For example, the EMS treated culture in the initial assay (Table 1) grew from  $3 \times 10^5$  cells/ml to  $1.059 \times 10^6$  cells/ml, a 3.53-fold increase, during the first 24 hours. The culture was then diluted to  $3 \times 10^5$  cells/ml and allowed to continue growing for another 24 hours. At that point, the cell concentration was  $1.320 \times 10^6$  cells/ml, a 4.4-fold increase. Thus the total growth was  $3.53 \times 4.4 = 15.4$ -fold increase over two days. Relative suspension growth (RSG) compares the treated groups against the appropriate negative controls. Absolute cloning efficiency (ACE) is the observed number of viable count clones compared to the expected number of 189 per plate ( $5.7 \text{ cells/ml} \times 33 \text{ ml} = 189 \text{ cells}$ ). Relative cloning efficiency (RCE) compares the treated groups with their respective negative controls. Thus, absolute cell survival (ACS) is the product of the suspension growth and absolute cloning efficiency while the relative cell survival (RCS) is the treated ACS compared to the control ACS.

Mutant Frequency

The mutant frequency (MF) is the mean selective plate count divided by the mean nonselective plate count multiplied by the dilution factor ( $2 \times 10^{-4}$ ). The dilution factor is derived from the ratio of the number of cells plated per ml in the nonselective plates divided by the number of cells plated per ml in the selective plates ( $5.7 / [2.86 \times 10^4] = 2 \times 10^{-4}$ ). The induced mutant frequency (IMF) in the treated groups is the observed mutant frequency less the spontaneous mutant frequency of the negative controls. Again, nonactivation and activation series are compared separately. The variance and standard error (SE) of the mutant frequency are calculated by using the mean selective and nonselective plate counts and with an assumed dilution variance of 10% (2,4).

Criteria for a Positive or Negative Response

An individual treatment concentration is considered positive if the assay is valid, the cell survival is at least 10% of controls, and the induced mutant frequency is at least three times ( $p < 0.01$ ) the standard error of that mutant frequency. A test compound is considered mutagenic if it

yields a correlated positive dose response through several (usually three) treatment concentrations (4-6).

A compound is considered nonmutagenic in this system if a valid assay does not yield a positive response and the limits of compound solubility (up to 5 mg/ml) or 90% reduction in cell survival has been reached. Normally, a determination of mutagenic potential is not made on the basis of only one assay. Both positive and negative assays are confirmed.

#### Deviations from the Protocol/SOP

In the initial assay, the concentration of 2AAF was 0.5 mg/ml instead of 0.05 mg/ml, which accounts for the extreme toxicity observed. This toxicity and the mutant frequency does, however, demonstrate that the activation system was functioning.

#### RESULTS

Nitroguanidine was assayed two times, one initial assay and one confirmatory assay. The compound exposure concentration and resulting data are presented in Tables 1 through 4, while the raw data are contained in Appendix B.

The initial assay (Tables 1 and 2) covered a dose range of 4 to 0.01 mg/ml. Absolute suspension growth and absolute cloning efficiency for the negative controls were within prescribed limits. The apparent greater-than-100% absolute cloning efficiency was the result of the time delay between cell counting and dilution of these samples. Both positive controls produced a statistically significant mutagenic response as required for a valid assay. The test compound, nitroguanidine, did not produce significant mutagenic activity at any concentration tested.

The confirmatory assay (Tables 3 and 4) covered a dose range of 4 to 1 mg/ml. This assay was also valid in terms of cell growth, cloning efficiency, and positive control-induced mutagenic activity. Again no statistically significant mutagenic activity was induced by nitroguanidine.



Table 1

## Cell Survival Data from the Initial Assay

Treatment			S-9 Cell Count		ASG <sup>a</sup>	RSG <sup>b</sup>	VCC <sup>c</sup>	ACE <sup>d</sup>	RCE <sup>e</sup>	RCS <sup>f</sup>	
			Day 1	Day 2							
			(x 10 <sup>3</sup> /ml)								
Control <sup>g</sup>			-	1032	1347	15.30	100	250	132	100	100
EMS	0.32	mg/ml	-	1059	1320	15.40	101	217	115	87	88
TP036	4.0	mg/ml	-	891	1254	12.60	82	276	146	110	90
TP036	2.0	mg/ml	-	848	1484	13.72	90	228	120	91	81
TP036	1.0	mg/ml	-	949	1337	14.40	94	245	129	98	92
TP036	0.5	mg/ml	-	994	1333	14.52	95	246	130	98	92
TP036	0.1	mg/ml	-	1029	1359	15.30	100	259	137	104	104
TP036	0.05	mg/ml	-	1155	1271	16.38	107	270	143	108	116
TP036	0.01	mg/ml	-	1112	1257	15.54	102	246	130	98	100
Control			+	880	1394	13.34	100	235	124	100	100
2AAF	0.5	mg/ml	+	77	136	0.45	3	28	15	12	0
TP036	3.66	mg/ml	+	756	1360	11.25	84	257	136	109	92
TP036	2.0	mg/ml	+	819	1299	11.61	87	268	142	114	100
TP036	1.0	mg/ml	+	867	1221	11.89	88	275	146	117	104
TP036	0.5	mg/ml	+	808	1394	12.42	93	250	132	106	99
TP036	0.1	mg/ml	+	906	1326	13.20	98	249	132	106	104
TP036	0.05	mg/ml	+	1002	1147	12.54	94	232	123	99	93
TP036	0.01	mg/ml	+	929	1127	11.78	86	307	162	131	113

a Absolute suspension growth = Total fold increase in suspension culture

b Relative suspension growth = treated/control x 100

c Viable Clone Count = mean of 3 plates

d Absolute Cloning Efficiency = viable clone count/189 x 100

e Relative Cloning Efficiency = treated/control x 100

f Relative Cell Survival = absolute cell survival

treated/absolute cell survival control x 100

g Mean values from both negative controls

Table 2

## Mutagenesis Data from the Initial Assay

Treatment			S-9	RCS <sup>a</sup>	MF <sup>b</sup> (x10 <sup>-6</sup> )	IMF <sup>c</sup> (x10 <sup>-6</sup> )	SE <sup>d</sup> (x10 <sup>-6</sup> )	IMF/SE <sup>e</sup>
Control <sup>f</sup>			-	100	23.9			
EMS	0.32	mg/ml	-	88	50.1	26.2	6.66	3.93
TP036	4.0	mg/ml	-	90	15.9	-7.9		
TP036	2.0	mg/ml	-	81	14.3	-9.5		
TP036	1.0	mg/ml	-	92	17.7	-6.2		
TP036	0.5	mg/ml	-	92	14.1	-9.8		
TP036	0.1	mg/ml	-	104	11.6	-12.3		
TP036	0.05	mg/ml	-	116	20.5	-3.4		
TP036	0.01	mg/ml	-	100	24.7	0.8	3.69	0.21
Control			+	100	46.0	0.0		
2AAF	0.5	mg/ml	+	0	178.6	132.6	33.52	3.96
TP036	3.66	mg/ml	+	92	34.0	-12.0		
TP036	2.0	mg/ml	+	100	30.8	-15.2		
TP036	1.0	mg/ml	+	104	30.0	-15.9		
TP036	0.5	mg/ml	+	99	38.5	-7.6		
TP036	0.1	mg/ml	+	104	24.9	-21.1		
TP036	0.05	mg/ml	+	93	33.3	-12.7		
TP036	0.01	mg/ml	+	113	41.1	-5.0		

a Relative Cell Survival = absolute cell survival  
treatment/absolute cell survival controls x 100

b Mutant Frequency = mutant clone count/viable clone count x  
dilution factor

c Induced Mutant Frequency = treated mutant frequency -  
control mutant frequency

d Standard Error is calculated only when the IMF > 0.

e Ratio of the IMF to SE > 3 indicates a positive treatment  
response

f Mean values from both negative controls

Table 3

## Cell Survival Data from the Confirmatory Assay

Treatment			S-9 <u>Cell Count</u>		ASG <sup>a</sup>	RSG <sup>b</sup>	VCC <sup>c</sup>	ACE <sup>d</sup>	RCE <sup>e</sup>	RCS <sup>f</sup>	
			Day 1	Day 2							
			(x 10 <sup>3</sup> /ml)								
Control <sup>g</sup>			-	1178	1662	21.65	100	169	89	100	100
EMS	0.32	mg/ml	-	705	1610	12.96	60	160	84	95	57
TP036	4.0	mg/ml	-	841	1576	14.84	69	177	94	105	72
TP036	2.0	mg/ml	-	1028	1657	18.70	86	163	86	97	83
TP036	1.0	mg/ml	-	1074	1649	19.80	91	172	91	102	93
Control <sup>g</sup>			+	793	1590	14.05	100	173	92	100	100
2AAF	0.05	mg/ml	+	589	1492	10.00	71	169	89	98	70
TP036	3.84	mg/ml	+	630	1552	10.92	78	157	83	91	71
TP036	2.0	mg/ml	+	689	1602	12.19	87	130	69	75	65
TP036	1.0	mg/ml	+	654	1632	11.88	85	157	83	91	77

<sup>a</sup> Absolute suspension growth = Total fold increase in suspension culture

<sup>b</sup> Relative suspension growth = treated/control x 100

<sup>c</sup> Viable Clone Count = mean of 3 plates

<sup>d</sup> Absolute Cloning Efficiency = viable clone count/189 x 100

<sup>e</sup> Relative Cloning Efficiency = treated/control x 100

<sup>f</sup> Relative Cell Survival = absolute cell survival treated/absolute cell survival control x 100

<sup>g</sup> Mean values from both negative controls

Table 4

## Mutagenesis Data from the Confirmatory Assay

Treatment			S-9	RCS <sup>a</sup>	MF <sup>b</sup> ( $\times 10^{-6}$ )	IMF <sup>c</sup> ( $10 \times 10^{-6}$ )	SE <sup>d</sup> ( $\times 10^{-6}$ )	IMF/SE <sup>e</sup>
Control <sup>f</sup>			-	100	60.8			
EMS	0.32	mg/ml	-	57	395.4	334.6	45.33	7.38
TP036	4.0	mg/ml	-	72	42.1	-18.7		
TP036	2.0	mg/ml	-	83	44.3	-16.5		
TP036	1.0	mg/ml	-	93	67.3	6.5	8.95	0.73
Control <sup>f</sup>			+	100	59.4			
2AAF	0.05	mg/ml	+	70	118.2	58.8	14.63	4.02
TP036	3.84	mg/ml	+	71	58.7	-0.7		
TP036	2.0	mg/ml	+	65	63.1	3.7	9.07	0.41
TP036	1.0	mg/ml	+	77	65.8	6.4	8.97	0.71

<sup>a</sup> Relative Cell Survival = absolute cell survival treatment/absolute cell survival controls  $\times 100$

<sup>b</sup> Mutant Frequency = mutant clone count/viable clone count  $\times$  dilution factor

<sup>c</sup> Induced Mutant Frequency = treated mutant frequency - control mutant frequency

<sup>d</sup> Standard Error of the mutant frequency calculated only when the IMF  $> 0$ .

<sup>e</sup> Ratio of the IMF to SE  $> 3$  indicates a positive treatment response

<sup>f</sup> Mean values from both negative controls

## DISCUSSION

The mutagenicity of nitroguanidine was evaluated in a mouse lymphoma forward mutation study consisting of an initial and a confirmatory assay. The results of this study indicated that nitroguanidine was not mutagenic in the mouse lymphoma test system. Both assays met the acceptance criteria for cloning efficiency, cell replication, positive control responses, and maximum test compound concentrations. The cloning efficiency of the negative controls without metabolic activation was greater than 70%. The total cell replication in suspension culture of these same controls was greater than 15 fold for the two days of culture. Both EMS and 2AAF induced statistically significant mutagenic responses. Spontaneous mutation rates were well within published values of  $25-115 \times 10^{-6}$  without activation and  $25-135 \times 10^{-6}$  with activation (3). The highest concentration of nitroguanidine tested was 4 mg/ml, which is nearly the limit of solubility for this compound.

Metabolic activation with rat liver S-9 did not significantly alter either the cytotoxicity or mutagenicity of nitroguanidine. Even at the highest doses, cytotoxicity was mild, with relative cell survivals of 70% or greater in all cases.

Nitroguanidine has been reported to cause significant chromosome damage in Chinese hamster fibroblasts (7). For this study, Ishidata and Odashima used a dose of 4 mg/ml for 24 hours and found 26% of the metaphases to have chromosomal aberrations, principally gaps, breaks and translocations. Our data from the CHO Sister Chromatid Exchange Assay (SCE) showed nitroguanidine to be very toxic at that dose over a 24-hour exposure (8). Most of the metaphases detected were from first division cells, and even those that showed some differential staining had not gone through two complete replication cycles in the BrdU-containing medium. Examination of the metaphases from this dose group did not reveal the chromosomal aberrations reported previously. Furthermore, lower concentrations that were less cytotoxic did not induce an increase in SCEs. Thus our data are at odds with those of Ishidata and Odashima (7) though the test systems were by no means identical (e.g. different cell lines, presence of BrdU, and different chromosome preparation).

Nitroguanidine was also negative in the Ames Salmonella/Mammalian Microsome Assay (8).

# CONCLUSION

The mutagenic potential of nitroguanidine was evaluated in the mouse lymphoma thymidine kinase forward mutation assay. Nitroguanidine was not mutagenic under conditions of this study.

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8. Harbell JW, Witcher LD, Sebastian SE, Korte DW. Studies on the mutagenic potential of nitroguanidine and nitrosoguanidine. Proceedings of the 1987 JANNAF Safety and Environmental Protection Subcommittee Meeting, 5-7 May 1987. Cleveland, Ohio. in press.

## Glossary

**Absolute Cell Survival:** The product of the population's total fold increase in growth in suspension culture multiplied by the absolute cloning efficiency under nonselective conditions.

**Absolute Cloning Efficiency:** The number of colonies counted on the nonselective plates divided by the number of cells originally plated  $\times 100$ .

**Fold Increase in Suspension Growth:** The quotient of the cell concentration at the end of the growth period divided by the starting cell concentration.

**Induced Mutant Frequency:** The mutant frequency of the treated population less the mutant frequency of the control population.

**Mutant Frequency:** The ratio of the number of colonies on the selective plates divided by the number of colonies on the nonselective plates multiplied by the dilution factor. The dilution factor is the ratio of the number of cells plated on the nonselective plates divided by the number plated on the selective plates.

**Relative Cell Survival:** The absolute cell survival of the treated population divided by the absolute cell survival of the negative control population  $\times 100$ .

**Relative Cloning Efficiency:** The absolute cloning efficiency of the treated population divided by the absolute cloning efficiency of the control population  $\times 100$ .

**Relative Suspension Growth:** Total fold increase in cell number during suspension growth of the treated population divided by the total fold increase in cell number during suspension growth of the negative control population  $\times 100$ .

**Total Fold Increase in Suspension Growth:** The product of the fold increase for the first day times the fold increase for the second day.



APPENDICES

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## CHEMICAL DATA

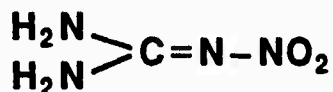
Chemical name: Nitroguanidine (NGu)

Other listed names: Guanidine, Nitro; alpha-Nitroguanidine;  
beta-Nitroguanidine

Chemical name: Nitroguanidine (NGu)

LAIR Code: TP036A

Structural formula:



Molecular formula:  $\text{CH}_4\text{N}_4\text{O}_2$

Molecular weight: 104.1

pH range of dosing suspensions: 6.7 - 7.4(1)

Physical state: White Powder

Melting point: 232°(2)

Source: Hercules Aerospace Division  
Sunflower Ammunition Plant  
DeSoto, Kansas

Lot No. SOW64K010-A-001

1. Wheeler CR. Nitrocellulose-Nitroguanidine Projects  
Laboratory Notebook #85-12-022, p 26. Letterman Army  
Institute of Research, Presidio of San Francisco, CA.
2. Fedoroff BT, Sheffield OE. Encyclopedia of explosives  
and related items. Vol V. Dover, New Jersey: Picatinny  
Arsenal 1975: G154.

Nitroguanidine Chemical Data (continued)

Analytical data/purity: The major peaks in the infrared spectrum of the compound were observed at 3450, 3396, 3342, 3278, 3201, 1666, 1634, 1525, 1404, 1314, 1151, 1045, 782  $\text{cm}^{-1}$  (3). The spectrum obtained for the test compound in our laboratory was identical to the Sadtler standard spectrum for nitroguanidine (4). HPLC showed only one peak (retention time 4.9 min) (5). The conditions employed were as follows: column, Brownlee RP-18 (4.6 x 250 mm); solvent 10% methanol/90% water, flow rate 0.7 ml/min; oven temperature, 50°C; monitoring wavelength, 265 nm.

3. Wheeler CR. Nitrocellulose-Nitroguanidine Projects. Laboratory Notebook #85-12-022, p. 22-23. Letterman Army Institute of Research, Presidio of San Francisco, CA.
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**Raw Data from the Initial Assay without Activation  
2 April 1985**

Treatment	S-9	Cell Counts Day 1 (x 10 <sup>3</sup> /ml)	Cell Counts Day 2 (x 10 <sup>3</sup> /ml)	Viable Clone Counts	Selective Clone Counts
Control	-	961 955 1010	1207 1196 1152	261 253 252	24 23 20
EMS 0.32 mg/ml	-	1078 1081 1017	1356 1308 1297	223 211 217	55 41 67
TP036 4.0 mg/ml	-	925 859 889	1321 1232 1209	291 263 274	23 20 23
TP036 2.0 mg/ml	-	845 852 847	1509 1472 1472	236 200 247	14 16 19
TP036 1.0 mg/ml	-	942 945 961	1341 1305 1364	252 252 230	13 27 25
TP036 0.5 mg/ml	-	953 1015 1013	1364 1345 1290	240 260 238	22 15 15
TP036 0.1 mg/ml	-	1015 1040 1032	1373 1394 1310	250 278 248	16 11 18
TP036 0.05 mg/ml	-	1149 1158 1159	1286 1277 1250	300 248 263	22 30 31
TP036 0.01 mg/ml	-	1130 1086 1120	1277 1232 1263	243 234 261	26 31 34
Neg	-	1077 1119 1022	1526 1493 1509	242 252 239	37 36 38

**Raw Data from the Initial Assay with Activation**  
**2 April 1985**

Treatment	S-9	Cell Counts Day 1 (x 10 <sup>3</sup> /ml)	Cell Counts Day 2 (x 10 <sup>3</sup> /ml)	Viable Clone Counts	Selective Clone Counts
Control	+	898 886 857	1418 1403 1361	227 245 233	53 60 49
2AAF 0.5 mg/ml	+	65 91 74	158 125 125	37 24 23	23 21 31
TP036 3.66 mg/ml	+	755 700 813	1360 1355 1365	260 260 250	43 44 44
TP036 2.0 mg/ml	+	856 799 803	1357 1297 1244	285 254 266	40 44 40
TP036 1.0 mg/ml	+	869 875 858	1200 1212 1251	279 263 284	32 44 48
TP036 0.5 mg/ml	+	781 827 815	1348 1418 1417	236 258 255	51 47 46
TP036 0.1 mg/ml	+	901 916 901	1322 1362 1295	238 266 243	23 35 35
TP036 0.05 mg/ml	+	997 1000 1010	1168 1152 1120	197 252 247	38 39 39
TP036 0.01 mg/ml	+	951 940 896	1140 1110 1132	320 307 293	73 58 58

**Raw Data from the Confirmatory Assay without  
Activation  
3 October 1985**

Treatment	S-9	Cell Counts Day 1 (x 10 <sup>3</sup> /ml)	Cell Counts Day 2 (x 10 <sup>3</sup> /ml)	Viable Clone Counts	Selective Clone Counts
Control	-	1135	1744	177	35
		1166	1745	159	44
		1146	1709	163	41
EMS    0.32 mg/ml	-	706	1597	144	294
		715	1594	149	320
		693	1640	186	333
TP036   4.0 mg/ml	-	837	1576	167	41
		865	1565	171	39
		820	1586	194	32
TP036   2.0 mg/ml	-	977	1674	157	25
		1075	1667	156	36
		1031	1629	179	48
TP036   1.0 mg/ml	-	1109	1695	162	60
		1024	1662	167	56
		1088	1590	188	58
Control	-	1226	1557	174	64
		1192	1591	172	58
		1202	1621	168	66

**Raw Data from the Confirmatory Assay with Activation  
3 October 1985**

Treatment	S-9	Cell Counts Day 1 (x 10 <sup>3</sup> /ml)	Cell Counts Day 2 (x 10 <sup>3</sup> /ml)	Viable Clone Counts	Selective Clone Counts
Control	+	777 736 736	1566 1546 1455	186 159 166	57 52 48
EMS 0.05 mg/ml	+	605 576 585	1483 1524 1470	162 169 175	90 104 105
TF036 3.84 mg/ml	+	641 612 637	1524 1582 1549	159 165 146	54 38 46
TP036 2.0 mg/ml	+	719 650 698	1560 1637 1609	133 137 120	40 38 45
TP036 1.0 mg/ml	+	657 642 664	1675 1612 1610	164 160 147	58 45 52
Control	+	853 831 823	1714 1668 1593	162 175 189	43 56 57

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